Example 5.4 **Oligosaccharide mapping by HPAEC-PAD (high-pH-anion exchange chromatography with pulsed amperometric detection)**

BioLC System, (Dionex, Sunnyvale) consisting of a AS50 Autosampler, AS50 Thermal Compartment, ED50 Electrochemical Detector, GS50 Gradient Pump, Software Chromeleon Chromatography Management System, was used along with a CarboPac PA-100 separation column (4 x 250 mm) and a CarboPac PA-100 pre-column (4 x 50 mm). Two different modes were used for the mapping and for quantitation of oligosaccharides.

Example 5.4(a) **HPAEC-PAD Asialo-mode:**

Neutral oligosaccharides were subjected to HPAEC-PAD mapping using a gradient of solvent A (200 mM NaOH) and solvent B (200 mM NaOH plus 600 mM Na-acetate) as depicted in the following table:

Table : Gradient for mapping of neutral oligosacharides

Time [min]	solvent A [%]	solvent B [%]
0	100	0
5	100	0
35	80	20
45	70	30
47	0	100
52	0	100
53	100	0
60	100	0

Flow rate : 1 ml / min

The detector potentials fort he electrochemical detector were

Table : Detector-Potentials for oligosaccharides

Time [ms]	potential [mV]
0	50
200	50
400	50
410	750

600	750
610	-150
1000	-150

Example 5.4(b) HPAEC-PAD Oligos-mode

Native oligosaccharides were subjected to HPAEC-PAD mapping using a gradient of solvent C (100 mM NaOH) and solvent D (100 mM NaOH plus 600 mM Na-acetate) as depicted in the following table

Table : Gradient mapping of native (sialylated) oligosaccharides

Time [min]	solvent C [%]	solvent D [%]
0	100	0
2	100	0
50	65	35
60	0	100
63	0	100
64	100	0
70	100	0

Flow rate : 1 ml / min

The detector potentials for the electrochemical detector were

Table : Detector-Potentials for oligosaccharides

Time [ms]	Potential [mV]
0	50
200	50
400	50
410	750
600	750
610	-150
1000	-150

The specific peak areas (nC x min x $nmol^{-1}$) were calculated using response factors obtained with defined oligosaccharide standards (disialylated diantennary, trisialylated triantennary, and terasialylated tetraantennary structures with and without N-

acetyllactosamine repeats all containing proximal alpha-1,6-linked fucose (Nimtz et al., 1993, Schroeter et al., 1999, Grabenhorst et al., 1999).

Example 5.5 Mass spectrometry of peptides and oligosaccharides

Example 5.5(a) Analysis by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI/TOF/TOF-MS

Intact glycoprotein preparations (chemically desialylated or enzymatically de-N-glycosylated forms) were analyzed with a Bruker ULTRAFLEX time-of-flight (TOF/TOF) instrument in the linear positive ion mode using a matrix of 22.4 mg 3,5-dimethoxy-4-hydroxy-cinnamic acid in 400 µl acetonitrile and 600 µl 0.1% (v/v) trifluoroacetic acid in H_2O; (glyco)-peptides were measured using a matrix of 19 mg alpha-cyano-4-hydroxycinnamic acid in the same solvent mixture using the reflectron for enhanced resolution. Native desialylated oligosaccharides were analyzed using 2,5-dihydroxybenzoic acid as UV-absorbing material in the positive as well as in the negative ion mode using the reflectron in both cases. For MS-MS analyses, selected parent ions were subjected to laser induced dissociation (LID) and the resulting fragment ions separated by the second TOF stage (LIFT) of the instrument. Sample solutions of 1 µl and an approximate concentration of 1-10 pmol·µl^{-1} were mixed with equal amounts of the respective matrix. This mixture was spotted onto a stainless steel target and dried at room temperature before analysis.

Example 5.5(b) Electrospray ionisation MS

1-3 µl aliquots of the tryptic digests corresponding to 2-20 pmol of protein were applied to a nanospray gold-coated glass capillary placed orthogonally in front of the entrance hole of a QTOF-II instrument (Micromass, UK). 1000 V were applied to the capillary and ions were separated by the time-of-flight analyser. For MS/MS analysis parent ions were selected by the quadrupole mass filter and subjected to collision induced dissociation. Resulting daughter ions were then separated by the TOF-analyzer. Spectra were processed by the MaxEnt3-programme (Micromass, UK) and the peptide sequence was determined using the PepSeq software.

Example 5.5(c) Compositional analysis of oligosaccharides from untreated and HES-modified EPO

Monosaccharides were analyzed as the corresponding methyl glycosides after methanolysis, N-reacetylation and trimethylsilylation by GC/MS [Chaplin, M. F. (1982) A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography; Anal Biochem. 1982 Jul 1;123(2):336-41]. The analyses were performed on a Finnigan GCQ ion trap mass spectrometer (Finnigan MAT corp., San Jose, CA) running in the positive ion EI mode equipped with a 30 m DB5 capillary column. Temperature program: 2 min isotherm at 80°C, then 10 degrees min^{-1} to 300 °C.

Monosaccharides were identified by their retention time and characteristic fragmentation pattern. The uncorrected results of electronic peak integration were used for quantification. Monosaccharides yielding more than one peak due to anomericity and/or the presence of furanoid and pyranoid forms were quantified by adding all major peaks. 2.0 µg of myo-inositol was added to samples and was used as an internal standard.

Example 6 Preparation of samples for mouse bioassay

HES-EPO conjugate samples, prepared according to Example 4 (2-3 mg /ml) were filtered through a 0.2 µm, Corning syringe filter unit (15 mm;RC membrane; Cat. No, 431215; Corning Incorporated, NY 14831). The samples were then frozen in liquid nitrogen in cryo vials and stored at -20°C until further use (see Example 7). EPO protein concentration was determined by UV-absorbance measurement at 280 nm according to European Pharmacopoeia, Fourth Edition, 2002, Directorate for the Quality of Medicines of the Council of Europe (EDQM).

Example 7 In-vivo assay of the biological activity of HES-modified EPO

The EPO-bioassay in the normocythaemic mouse system was performed according to the procedures described in the European Pharmacopeia 4, Monography 01/2002:1316 on the basis of the HES-EPO prepared according to Example 6: Erythropoietin concentrated solution and Ph. Eur. Chapter 5.3 : "Statistical Analysis of Results of Biological Assays

and Tests"; in deviation from this assay the laboratory that carried out the EPO assay was using the international BRP EPO reference standard preparation in a 4-fold dilution. Therefore it was necessary to divide the received results by 4.

For the HES-modified EPO 50/0.7 a value for the specific activity of 533 000 units per mg EPO of protein was measured indicating an approximately 4-5 fold higher specific activity when compared to the EPO starting material. The results of the study are summarized in the following table:

Sample description	**Value** (normocythaemic mouse assay)	**Value / 4** (normocythaemic mouse assay)	**Calculated specific activity of EPO sample** (based on A280 and RP-HPLC determination)
EPO starting material (not modified)	354	89	115 900 U/mg
EPO-HydroxylaminoHES10/0.4	178	45	117 000 U/mg
EPO-HydroxylaminoHES10/0.7	459	115	299 000 U/mg
EPO-HydroxylaminoHES50/0.4	228	57	149 700 U/mg
EPO starting material (incubated with HES10/0.4; per-ox)	208	52	67 600 U/mg
EPO HydroxylaminoHES50/0.7	821	205	533 000 U/mg

Results

Results 1 Purification of EPO and modified EPO forms by anion exchange chromatography on Q-Sepharose.

4 mg quantities of EPO, periodate oxidised EPO and HES-modified EPO forms were subjected to anion exchange chromatography on Q-Sepharose as described under Example 4. After buffer exchange to 50 mM Na-phosphate buffer pH 7.2 aliquots of samples were subjected to de-*N*-glycosylation by polypeptide N-glycosidase (PNGase) treatment (see Example 5.1). 5-10 µg aliquots of samples before and after PNGase treatment were subjected to SDS-PAGE analysis. As is depicted in Figure 1, the samples A14 (= EPO) and

A24 (periodate oxidised EPO incubated with unmodified HES) yielded the O-glycosylated EPO form after incubation with PNGase; whereas for samples A20, A21, A23 and A25 an additional diffuse Coomassie stained band was detected (indicated by brackets in Figure 1) which represents EPO forms that are modified with HES at their O-glycan.

Results 2 **RP-HPLC separation of liberated N-glycans and de-N-glycosylated EPO polypeptide**

The de-N-glycosylated EPO forms were separated from liberated N-glycans by RP-HPLC as described in Example 5.2) and the resulting oligosaccharide fractions and the EPO protein were subjected to further analysis.

After SDS-PAGE analysis the EPO forms which were modified by HAS at their O-glycans (see Figure 2) disappeared after mild acid treatment of the de-N-glycosylated samples which were previously isolated by RP-HPLC (see Example 5.2) indicating the acid labile nature of the modification (removal of sialic acid from the O-glycosylated EPO form by mild acid treatment is also indicated by the small shift of the non-hasylated protein band.

Results 3 **Oligosaccharide characterisation of unmodified and HAS-modified EPO preparations**

The oligosaccharide fractions obtained after RP-HPLC of PNGase-treated EPO forms were desalted and aliquots corresponding to 1 -3 nmoles were subjected to HPAEC-PAD analysis and to compositional analysis as described under Example 5.5(c).

Results 3(a) **HPAEC-PAD Mapping of native N-glycans**

Mapping of the N-glycans of untreated EPO yielded an oligosaccharide pattern as depicted in Figure 4. Based on peak response in HPAEC-PAD 0,7 % of the oligosaccharide peak area was detected in the region of monosialylated glycans (22-25 min), 7 % disialylated (28-32,5 min), (4 % Man_6-P= 34,5 min), 30 % trisialylated (36-41 min) and 58 % tetrasialylated glycans (42-50 min).

After mild periodate oxidation of EPO the resulting N-glycans eluted as follows: 1,2 % in the monosialo, 6.0 in the disialo, 2 % in the Man6-P, 12% in the trisialo and 26 % in the tetrasialo region whereas 50% of the HPAEC-PAD signal detected was observed at 51-57 min.

ESI-MS of the reduced and permethylated oligosaccharides indicated that about 80-85 % of the N-acetylneuraminic acids were modified by periodate treatment (data not shown).

In the case of the oligosaccharide material obtained from HAS modified EPO preparations 90% of the HPAEC-PAD signal was detected at a retention time of 52-59 minutes (using a gradient as described in Example 5.4(b)) thus indicating that periodate oxidised EPO was HAS modified at almost completely all N-glycosylation sites.

Results 3(b) HPAEC-PAD Mapping of N-glycans after mild acid treatment (see Figure 5)

Upon mild acid treatment (see Example 5.3) the oligosaccharides of EPO sample A14 yielded di-tri- and tetraantennary oligosaccharide signals which were identified by comparison of their retention time with standard reference complex-type structures. The %-peak areas for individual glycans structures are depicted in the following table:

Table: Individual asialo oligosaccharide structures (% peak area) after mild acid treatment observed in HPAEC-PAD analysis

oligosaccharide-structure	A14 (% peak area)	A20 (% peak area)	A21 (% peak area))	A23 (% peak area)	A24 (% peak area)	A25 (% peak area)
diantennary	2,8	3,0	3,0	2,9	2,1	2,6
2,4-triantennary	2,8	3,0	2,9	3,0	2,4	2,8
2,6-triantennäay	6,0	6,3	6,2	6,2	5,5	6,0
Tetraantennary	33,5	33,2	33,5	35,0	33,9	34,5
Triantennary+1R	5,5	6,2	6,0	6,3	5,7	6,4
Tetraantennary+1R	22,7	22,5	22,2	23,1	22,9	22,9

Triantennary+2R	2,0	2,5	2,3	2,5	2,4	2,6
Tetraantennary+2R	9,3	9,1	8,9	9,2	9,3	9,1
Triantennary+3R	0,5	0,5	0,5	0,5	0,7	0,6
Tetraantennary+3R	2,2	2,4	2,4	2,1	2,8	2,1
Man_6-Phosphat	2,8	2,4	2,8	2,2	1,9	1,8
Residual monosialylated oligosaccharides*	10,2	9,1	9,3	6,9	10,4	8,6

* due to the presence of O-acetylated N-acetylneuraminic acid residues which are partially resistent to mild acid treatment (see Fig.5 indicated by a bracket, trace 2 from the top).

In summary, the starting material (A14), the mock HES incubated periodate oxidised EPO (A24) and the HES-modified EPO preparations exhibited an identical glycan pattern indicating that the derivatization procedures did not significantly affect the neutral carbohydrate structures. This was further confirmed by MALDI/TOF MS of the mild acid treated oligosaccharides revealing molecular ion signals at m = 1809 (diantennary + proximal Fucose), m= 2174 (triantennary + proximal Fucose), m= 2539 (tetraantennary + proximal Fucose), m= 2904 (tetraantennary + 1 N-acetyllactosamin repeat + proximal Fucose), m= 3269 (tetraantennary + 2 N-acetyllactosamin repeat + proximal Fucose).

Results 3(c) Compositional analysis of native N-glycans

Native oligosaccharides of EPO isolated by RP-HPLC (see Example 5.2) were reduced and derivatised as described under Example 5.5(c). The following table compares the monosaccharide composition of the oligosaccharides after de-N-glycosylation of EPO preparations yielding a fucose, mannose galactose and N-acetylglucosamine ratio of approximately 1: 3 : 3.5 : 3 (values for GlcNAc-derivatives were low due to loss during the derivatisation procedure). The amount of the derivative for N-acetylneuraminic acid (NeuAc) detected in compositional analysis is in agreement with the amount of intact NeuAc observed in the desialylated N-glycan preparations in HPAEC-PA mapping.

Table: Compositional analyses of N-glycans from hasylated EPO-preparations A14, A20-A25

Sample:	Fuc	Man	Gal	Glc	GlcNAc	GlcHe1	GlcHe2	NeuNAc	Inositol
A14	1.17	3.00	3.23	---	2.33	----	----	0.74	6.83
A21	1.10	3.00	3.61	15.81	2.79	6.51	1.03	0.90	9.85
A23	1.03	3.00	3.85	73.78	2.16	32.08	5.15	0.92	13.41
A24	0.68	3.00	3.40	---	2.55	---	---	0.62	9.08
A20	1.04	3.00	3.13	43.5	2.99	2.74	1.07	1.11	3.27
A21	1.01	3.00	2.40	15.05	1.98	3.68	1.47	0.84	2.01
A25	1.18	3.00	2.86	148.9	1.90	40.99	1.99	0.78	2.02

Uncorrected results of the electronic integration of the peaks of the pertrimethylsilylated monosaccharide methylglycosides. 2.0 µg of Inositol was added to the samples as internal standard.

Information Sample code

0312-17/**A14**	=	EPO = starting material prior to periodate oxidation
0401-09/**A20**	=	HES 10/04 periodate-oxidated
0401-09/**A21**	=	HES 10/07 periodate-oxidated
0401-09/**A24**	=	A14/mock-HES periodate-oxidated
0401-09/**A23**	=	HES 50/07 periodate-oxidated
0401-09/**A25**	=	HES 50/04 periodate-oxidated

The detection of glucose and its mono- and di-hydroxethylated derivatives in the oligosaccharide preparations of A20, A21, A23 and A 25 confirmed the presence of HAS in this material and was not detected in the starting material (A14) or the material from EPO incubated with underivatised HES (A24).

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Claims

1. A method of producing a conjugate of erythropoietin and hydroxyethyl starch, said method comprising reacting the hydroxyethyl starch with a crosslinking compound having two hydroxylamino groups to give a hydroxylamino functionalized hydroxyethyl starch derivative, and reacting the hydroxylamino group of said derivative with a carbohydrate moiety of the erythropoietin, wherein the hydroxyethyl starch has a mean molecular weight of at least 40 kD and a degree of substitution of at least 0.6.

2. The method as claimed in claim 1, wherein the hydroxyethyl starch has a mean molecular weight of at least 50 kD and a degree of substitution of at least 0.7.

3. The method as claimed in claim 1 or 2, wherein the hydroxyethyl starch has a mean molecular weight of about 50 kD and a degree of substitution of about 0.7 to 0.8.

4. The method as claimed in any of claims 1 to 3, wherein the crosslinking compound is O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine.

5. The method as claimed in any of claims 1 to 4, wherein the hydroxyethyl starch is reacted with the crosslinking compound in an aqueous medium.

6. The method as claimed in any of claims 1 to 5, wherein the reaction is carried out at a pH of from 4.5 to 6.5.

7. The method as claimed in any of claims 1 to 6, wherein the reaction is carried out at a temperature of from 20 to 25 °C.

8. The method as claimed in any of claims 1 to 7, wherein the hydroxylamino functionalized hydroxyethyl starch derivative is reacted with the carbohydrate moiety of the erythropoietin in an aqueous medium.

9. The method as claimed in claim 8, wherein the reaction is carried out at a pH of from 4.5 to 6.5.

10. The method as claimed in claim 8 or 9, wherein the reaction is carried out at a temperature of from 20 to 25 °C.

11. The method as claimed in any of claims 1 to 10, wherein the carbohydrate moiety is an oxidized terminal saccharide unit of a carbohydrate side chain of the erythropoietin, preferably a terminal sialic acid.

12. The method as claimed in claim 11, wherein the terminal saccharide unit is oxidized, optionally after partial or complete enzymatic and/or chemical removal of the terminal sialic acid.

13. The method as claimed in claim 11 or 12, wherein the terminal saccharide unit is galactose.

14. The method as claimed in any of claims 1 to 13, wherein the carbohydrate moiety is comprised in a carbohydrate side chain of the erythropoietin which was attached to the erythropoietin via N- and/or O-linked glycosylation during its production in mammalian, especially human cells, insect cells, or yeast cells.

15. A conjugate of erythropoietin and hydroxyethyl starch, obtainable by a method as claimed in any of claims 1 to 14.

16. A conjugate comprising hydroxyethyl starch, a crosslinking compound and erythropoietin, wherein the crosslinking compound is linked via an oxime linkage and/or an oxyamino group to the hydroxyethyl starch and via an oxime linkage to the carbohydrate moiety of the erythropoietin, and wherein the hydroxyethyl starch has a mean molecular weight of at least 40 kD and a degree of substitution of at least 0.6.

17. The conjugate as claimed in claim 16, wherein the hydroxyethyl starch has a mean molecular weight of at least 50 kD and a degree of substitution of at least 0.7.

18. The conjugate as claimed in claim 16 or 17, wherein the hydroxyethyl starch has a mean molecular weight of about 50 kD and a degree of substitution of about 0.7 to about 0.8.

19. The conjugate as claimed in any of claims 16 to 18, comprising a crosslinking compound having had two hydroxylamino groups, one of which being covalently linked to a carbohydrate moiety of the erythropoietin and one of which being covalently linked to the hydroxyethyl starch.

20. The conjugate as claimed in any of claims 16 to 19, wherein the crosslinking compound is O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine.

21. The conjugate as claimed in any of claims 16 to 20, wherein the erythropoietin has the amino acid sequence of human erythropoietin.

22. The conjugate as claimed in any of claims 16 to 21, wherein the carbohydrate moiety is comprised in a carbohydrate side chain attached to the erythropoietin via N- and/or O-linked glycosylation, the erythropoietin comprising at least one carbohydrate side chain.

23. The conjugate as claimed in claim 22, wherein the at least one carbohydrate side chain was attached to the erythropoietin during the production of the erythropoietin in mammalian, especially human cells, insect cells, yeast cells, transgenic animals or transgenic plants.

24. The conjugate as claimed in any of claims 16 to 23, wherein the carbohydrate moiety is an oxidized galactose residue or a sialic acid residue.

25. A method for screening for a conjugate of erythropoietin and hydroxyalkyl starch, preferably hydroxyethyl starch, having improved in vivo activity compared to native erythropoietin, said method comprising the steps of

(i) providing a candidate conjugate;

(ii) testing the in vivo activity in comparison with native erythropoietin,

wherein the mean molecular weight MW is varied in the range of from 1 to 300 kD and the degree of substitution DS is varied in the range of from 0.1 to 1.0, and wherein these parameters are simultaneously increased compared to a given combination of parameters.

26. The method as claimed in claim 25, wherein the given combination of parameters is a mean molecular weight MW of about 10 kD and a degree of substitution DS of about 0.4.

27. The method as claimed in claim 25 or 26, further comprising the step of incorporating the candidate conjugate into a therapeutic or prophylactic composition.

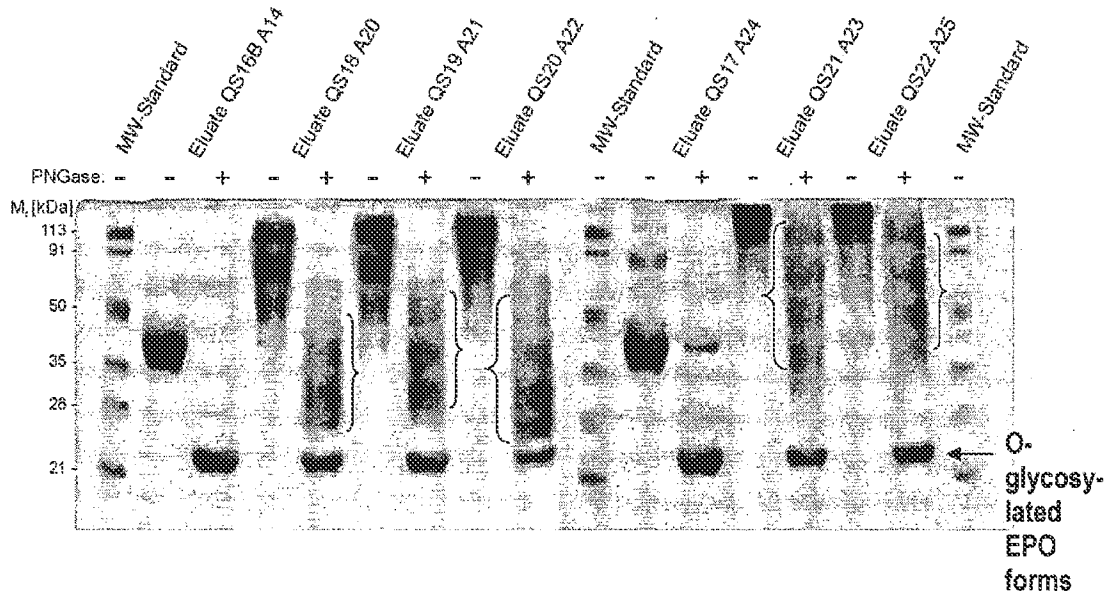
28. A conjugate as claimed in any of claims 15 to 24 for use in a method for the treatment of the human or animal body.

29. A pharmaceutical composition comprising in a therapeutically effective amount a conjugate as claimed in any of claims 15 to 24.

30. A pharmaceutical composition as claimed in claim 29, further comprising at least one pharmaceutically acceptable diluent, adjuvant, and/or carrier.

31. Use of a conjugate as claimed in any of claims 15 to 24 for the preparation of a medicament for the treatment of anemic disorders or hematopoietic dysfunction disorders or diseases related thereto.

Fig. 1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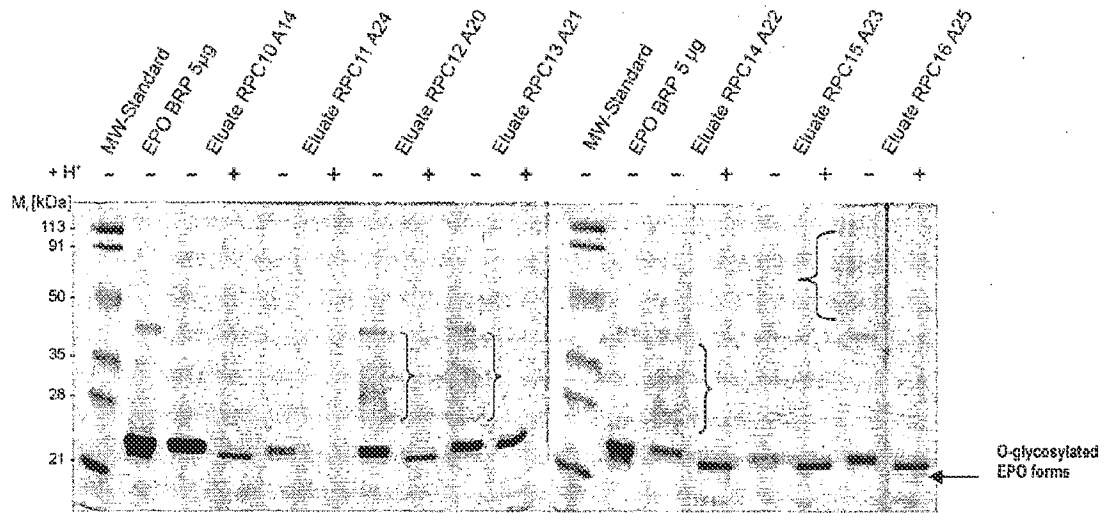
Eluate QS16 A20
Eluate QS19 A21
Eluate QS20 A22
MW-Standard
Eluate QS17 A24
Eluate QS21 A23
Eluate QS22 A25
MW-Standard
PNGase: - - + - + - + - + - - + - + - + -
M, [kDa]
113
91
50
35
28
21
O-glycosylated EPO forms

Fig.2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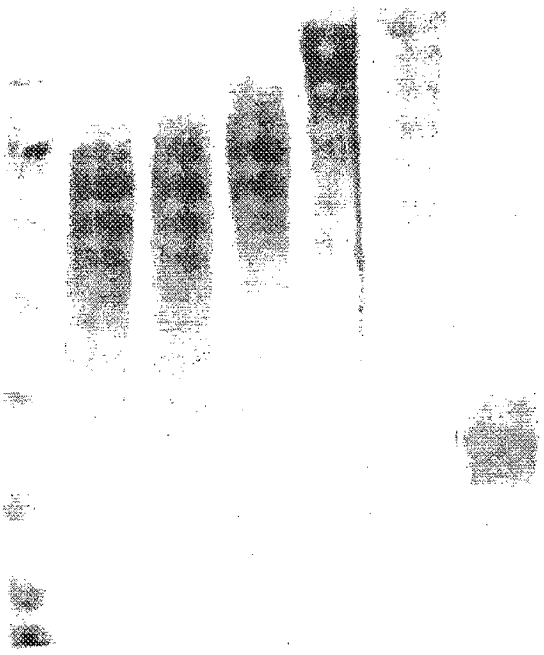
Eluate RPC10 A14
Eluate RPC11 A24
Eluate RPC12 A20
Eluate RPC13 A21
MW-Standard
EPO BRP 5 µg
Eluate RPC14 A22
Eluate RPC15 A23
Eluate RPC16 A25
+ H+
M, [kDa]
113
91
50
35
28
21
O-glycosylated
EPO forms

Fig. 3



A B C D E F G

Fig.4

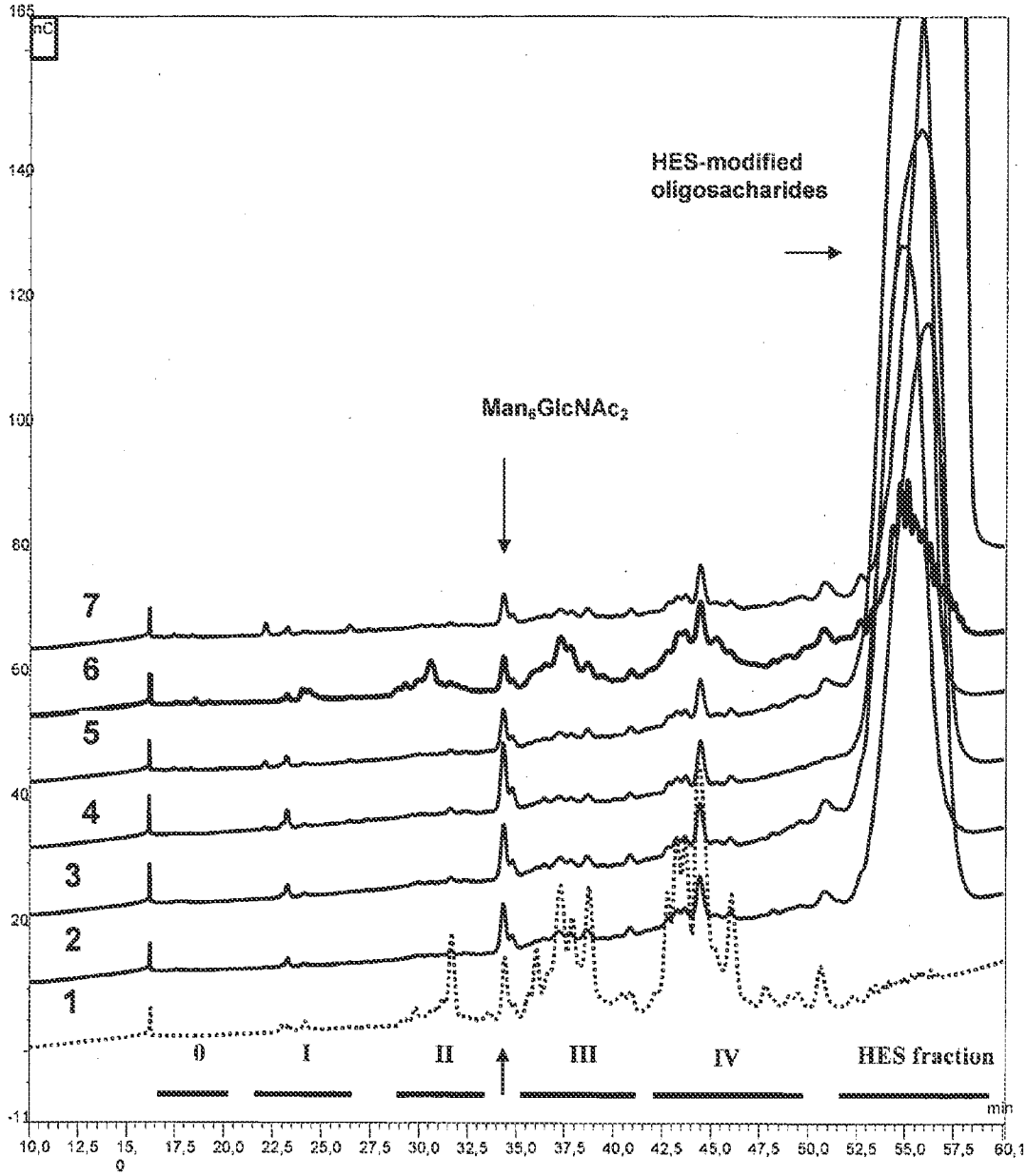
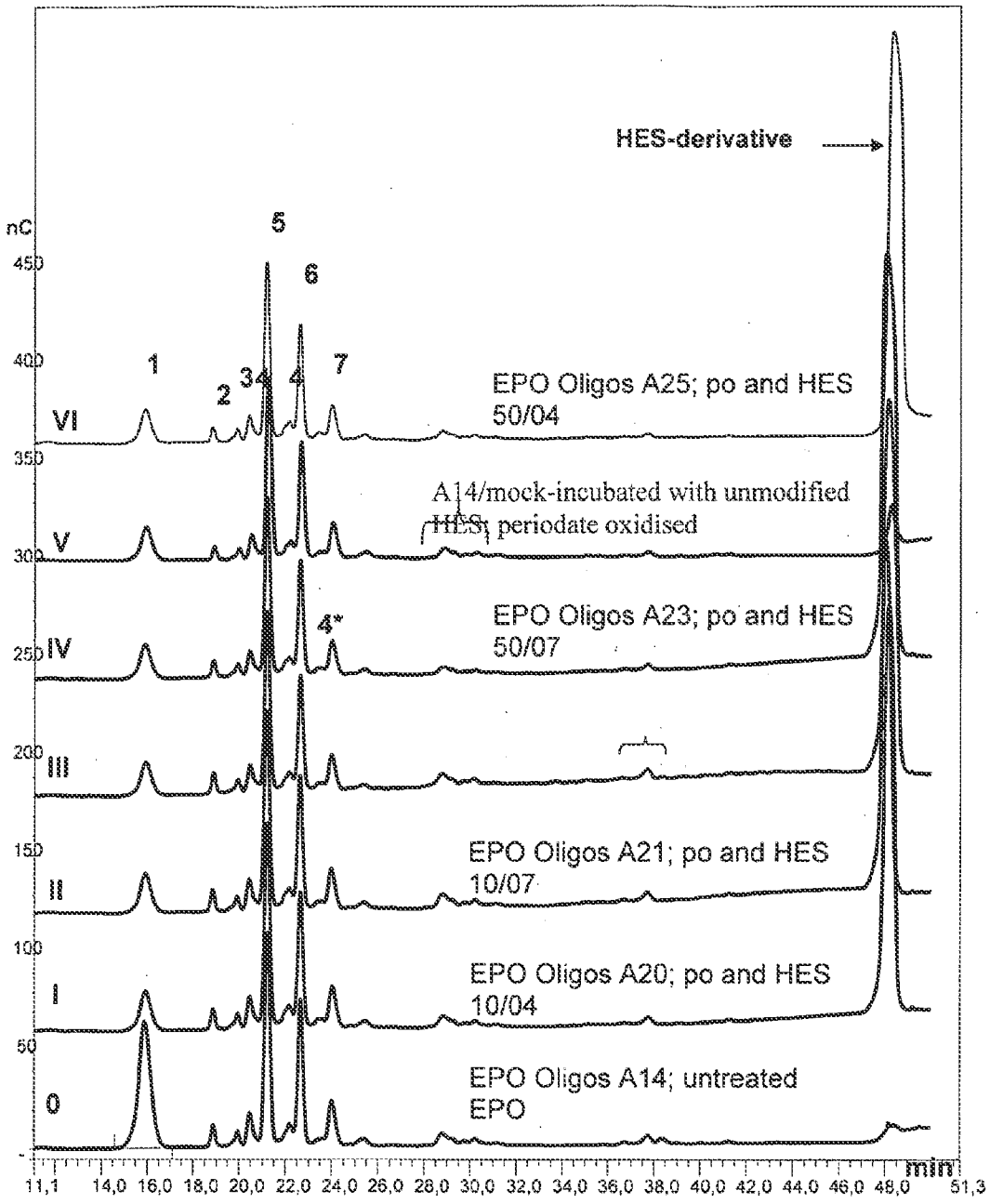

nC
HES-modified
oligosacharides
Man5GlcNAc2
7
6
5
4
3
2
1
0
I
II
III
IV
HES fraction
min

Fig. 5


HES-derivative
nC
450
400
350
300
250
200
150
100
50
-
5
6
1
2
3
4
7
VI
EPO Oligos A25; po and HES 50/04
A14/mock-incubated with unmodified HES; periodate oxidised
V
4*
IV
EPO Oligos A23; po and HES 50/07
III
II
EPO Oligos A21; po and HES 10/07
I
EPO Oligos A20; po and HES 10/04
0
EPO Oligos A14; untreated EPO
min
11,1 14,0 16,0 18,0 20,0 22,0 24,0 26,0 28,0 30,0 32,0 34,0 36,0 38,0 40,0 42,0 44,0 46,0 48,0 51,3